PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 27/447	A1	(11) International Publication Number: WO 00/16087
		(43) International Publication Date: 23 March 2000 (23.03.00)

(21) International Application Number: PCT/US99/20836

(22) International Filing Date: 9 September 1999 (09.09.99)

(30) Priority Data:
09/154,178   16 September 1998 (16.09.98)   US

(71) Applicant: THE PERKIN–ELMER CORPORATION [US/US]; 850 Lincoln Centre Drive, Foster City, CA 94404 (US).

(72) Inventors: SHARAF, Muhammad, A.; 96 Sequoyah View Drive, Oakland, CA 94605 (US). ROQUE–BIEWER, Maria, C.; 4600 Susan Drive, #203, San Bruno, CA 94066 (US).

(74) Agent: BORTNER, Scott, R.; The Perkin–Elmer Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SPECTRAL CALIBRATION OF FLUORESCENT POLYNUCLEOTIDE SEPARATION APPARATUS

(57) Abstract

The invention relates to methods, compositions, and systems for calibrating a fluorescent polynucleotide separation apparatus. One aspect of the invention is multiple color calibration standards and their use. A multiple color calibration standard is a mixture of at least two polynucleotides of different length, wherein each of the polynucleotides is labelled with a spectrally distinct fluorescent dye. Another aspect of the invention is to produce total emission temporal profiles of multiple color calibration standards for use in calibrating fluorescent polynucleotide separation apparatus. The peaks corresponding to the fluorescently labelled polynucleotide in the total emission temporal profile may be detected using a peak detector that is driven by changes in the slopes of the total emission temporal profile. Calibration of fluorescent polynucleotide separation apparatus with various embodiments of the methods of the invention includes the step of identification of the labelled polynucleotide of the multiple color calibration standards. The process of spectral calibration of fluorescent polynucleotide separation apparatus using multiple color calibration standard may include the step of the estimating (extracting) of the dyes' reference spectra, using information from the peak detection process performed on the total emission temporal profile. Other aspects of the invention include systems for separating and detecting fluorescently labelled polynucleotide, wherein the system is designed for spectral calibration in accordance with the subject calibration methods employing multiple color calibration standards. Another aspect of the invention is methods and compositions for detecting the flow of electrical current through a separation channel of a fluorescent polynucleotide separation apparatus. These methods and compositions employ monitoring dyes. Monitoring dyes are fluorescent dyes that are spectrally distinct from the dye on the polynucleotide intended to convey genetic information, e.g., fluorescent polynucleotide sequencing reaction products.

SPECTRAL CALIBRATION OF FLUORESCENT
POLYNUCLEOTIDE SEPARATION APPARATUS

Field of the Invention

The invention in the field of spectral calibration of fluorescence based automated polynucleotide length measurement instruments.

5   Background

Spectral calibration is to estimate reference spectral profiles (reference spectra) of particular fluorescent dyes using the optical measurement system of a automated DNA sequencer or similar fluorescent polynucleotide separation apparatus where the particular dyes will be utilized. The current practice of spectral calibration relies on measuring the spectral

10   profile of each fluorescent dye separately. This approach to spectral calibration of fluorescent polynucleotide separation apparatus results in reduced throughput because it requires N lanes on gel-based instruments and requires N separate runs on capillary-based instrument. As more fluorescent dyes are developed and utilized routinely (N is expected to increase,) the spectral calibration of fluorescent polynucleotide separation apparatus becomes more demanding and less

15   efficient under the current practice. Additionally, the amount of computer resources devoted to spectral calibration also increases with the number of dyes and separation channels analyzed.

Summary

The invention relates to methods, compositions, and systems for calibrating a

20   fluorescent polynucleotide separation apparatus. Fluorescent polynucleotide separation apparatus, such as automated DNA sequencer, must be spectrally calibrated for use with the different fluorescent dyes to be used in conjunction with the separation system.

One aspect of the invention is multiple color calibration standards and their use. A multiple color calibration standard is a mixture of at least two polynucleotide of different length,

25   wherein each of the polynucleotide is labeled with a spectrally distinct fluorescent dye. In a preferred embodiments of the invention, the multiple color calibration standard comprise at least four polynucleotide of different length, and each of the polynucleotide is labeled with a spectrally distinct dye. The invention includes numerous methods of spectrally calibrating a fluorescent polynucleotide separation apparatus with a multiple color calibration standard.

Definitions

The term *"fluorescent polynucleotide separation apparatus"* as used herein denotes an apparatus for separating fluorescently labeled polynucleotide mixtures (*e.g.* by electrophoresis) and detecting the separated polynucleotides by the fluorescence emission produced from exciting the fluorescent dye. Examples of fluorescent polynucleotide separation apparatus include automated DNA sequencers such as the PE Applied Biosystems 310 and 377 (Foster City, California). Examples of fluorescent polynucleotide separation apparatus are also described in, among other places, U.S. Patents Nos. 4,971,677; 5,062,942; 5,213,673; 5,277,780; 5,307,148; 4,811,218; and 5,274,240. The term fluorescent polynucleotide separation apparatus also includes similar instruments for polynucleotide fragment length analysis that are not capable of the single base pair resolution required to obtain DNA base sequence information. Fluorescent polynucleotide separation apparatus comprises one or more separation regions or channels, typically the path of electric current flow in electrophoretic separation devices. Types of separation channels include capillaries, microchannels, tubes, slab gels, and the like. Fluorescent polynucleotide separation apparatus collect several types of data during their operation. This data includes spectral data and temporal data relating to the fluorescent labeled polynucleotides separated by the apparatus. Typically, such data is collected by a detector (*e.g.* a CCD array, photomultiplier tubes, and the like) designed to obtain quantitative spectral data over a predetermined region or regions of the separation channels. Spectral data collected by the apparatus includes the intensity of fluorescence at a plurality of wavelengths. The different wavelengths sampled are referred to as bins or channels. The apparatus also collects temporal data that is correlated with the spectral data. The temporal data is collected at numerous different time points. For example, a detector at a fixed position will measure increases and decreases in fluorescence intensity as a function of time as a labeled polynucleotide peak passes by the detector. This temporal data may be expressed as "frame" or "scan" number to indicate the different temporal sampling points.

A *temporal profile* is a plot of the intensity of a spectral signal as a function of time or scan/frame number. A temporal profile consists of systematic and random variations. *Systematic variations* are caused by peaks, spikes and background drifts. These variations cause the shape of the profile to undergo specific, and often predictable, changes. By contrast, *random variations* do not cause specific or predictable changes in the temporal profile. A temporal profile

parameter of the peak detector. If a peak is actually present, the threshold value is also used to indicate that the temporal profile has returned to baseline levels and that the peak has ended.

Peak start is the first point along the peak segment of a temporal profile. A peak start may be found at baseline levels, or in the valley between two peaks. *Peak end* is the last point
5 along the peak segment of a temporal profile. A peak end may be found at baseline levels, or in the valley between two peaks. *Peak maximum* is a point along the peak segment of a profile where the highest intensity is found. *Peak width* is the number of data points between the start of the peak and the end of the peak (see Figure 1.) The peak width attribute is helpful in discriminating between peaks that correspond to labeled DNA fragments and spikes. The latter
10 have relatively smaller peak widths.

Peak height at maximum is the intensity at peak maximum corrected for the analytical background (see Figure 1.) *Peak S/N ratio* refers to the ratio of the peak height at maximum to the analytical noise of the temporal profile. A peak's S/N attribute is an effective parameter that is used to retain the peak information of the dye-labeled fragments of the multiple color
15 calibration standard.

Migration time of a peak is the time elapsed from the start of the electrophoresis to peak maximum. A particular peak corresponding to a certain labeled polynucleotide of the multiple color calibration standard may serve as a *reference peak* whose migration time is a reference point from which the migration time of other peaks are measured.

20 *Migration time offset* is the difference between the migration time of a particular peak and the migration time of the reference peak (see Figure 1.) Peaks to the left of the reference peak will have negative migration time offsets, while those to the right of the reference peak will have positive migration time offsets. Reference peaks are located based on rank or migration time. Subsequently, migration time offsets are used to locate all other dye-labeled fragments.

25 *Input parameters* are attributes that are used by a particular implementation of the algorithm. These parameters may be specific to the multiple color calibration standard as well as to the platform being used. The implementation attributes may include the peak width, the threshold variable, the peak S/N ratio, the reference peak locator (migration time *vs.* rank) the migration time offsets, and the appropriate tolerances, if necessary, to account for instrumental
30 and experimental variations.

A total emission temporal profile is a sum of the intensities of the fluorescence signal obtained in all spectral channels as a function of time. Peaks corresponding to the different oligonucleotides in the multiple color calibration standard may then be determined by analyzing the total emission temporal profile with a peak detection transformation function. A reference

5   spectrum for each of the fluorescent dye of interest used in the multiple color calibration standard may then be produced by selecting a reference spectrum that substantially corresponds to the relevant peak of the total emission profile.

        Other aspects of the invention are multiple color calibration standards and their use. A multiple color calibration standard is a mixture of at least two polynucleotides of different length.

10   (It will be understood by person skilled in the art that each polynucleotide is present in a large number of essentially identical copies so as to provide useful amounts of the subject compositions) Preferably, the length (in number of bases) of each labeled polynucleotide is known precisely so as to maximize the accuracy of the standard. Each of the different length polynucleotides in the standard is labeled with a different fluorescent dye. The predetermined

15   correlation between the length of the given polynucleotide and the particular fluorescent dye that is attached to that polynucleotide is used to identify the polynucleotide of the multiple color calibration standard during the calibration process. The different fluorescent dyes are selected so as to have distinctive spectral profiles (for the same excitation frequency). Preferably the sizes of the polynucleotide in the multiple color calibration standard are selected so as to ensure

20   sufficient separation between the polynucleotide labeled with different dyes that the spectral profile peaks of the fluorescent dyes do not significantly overlap. In other words, there is preferably sufficient difference between the lengths of the constituent polynucleotides so that for any given polynucleotide peak that is being detected, the possibility that the fluorescence intensity readings are the result of multiple different dyes is minimal.

25   The sizes of the polynucleotides that are in multiple color calibration standards are selected so as to be within the size separation for the particular fluorescent polynucleotide separation apparatus for which they are designed to be used. Exemplary of such a range is about 10 - 1500 bases in length, preferably about 10 - 1000 bases in length, more preferably about 20 - 500 bases in length. Preferably polynucleotides in the standard are separated by at least 10 bases

30   in length. Methods of making the polynucleotide components of the subject standards are well known to persons of ordinary skill in the art. Such methods include the complete in vitro

dichlorofluorescein, 2',7'-dimethoxy-4',5'-6-carboxyrhodamine (JOE), N',N',N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) and 6-carboxy-X-rhodamine (ROX). Fluorescent dyes are described in, among other places, U.S. patent 4,855,225; Menchen et al, U.S. patent 5,188,934; Bergot et al, International Application PCT/US90/05565; Haugland, R.P.,Handbook of

5   Fluorescent Probe and Research Chemicals , 6th edition (1996) and like references. Examples of such dyes include methods of attaching fluorescent dyes to polynucleotides are also well known to those skilled in the art. Examples of such attachment methods can be found in, among other places, U.S. Patent Nos. 4,789,737; 4,876,335; 4,820,812; and 4,667,025.

        The multiple color calibration standards of the invention may also comprise various

10   other components in addition to fluorescent labeled polynucleotides. Such additional components may be used to improve the movement of the polynucleotide through a separation channel of a flour scent polynucleotide separation apparatus. Examples of additional components include, but are not limited to, buffers, denaturants, and the like.

        The invention includes numerous methods of spectrally calibrating a fluorescent

15   polynucleotide separation apparatus with a multiple color calibration standard. A multiple color calibration standard is introduced, i.e., loaded, into a fluorescent polynucleotide separation apparatus. The introduction of a multiple color calibration standard into a florescent polynucleotide separation apparatus and the subsequent separation of the component of the standard along with the collection of the spectral and temporal data obtained from detecting the

20   separated labeled polynucleotides may be conveniently referred to as producing a spectral calibration run. Spectral calibration run may runs may be performed on a single separation channel or may be simultaneously performed on serval separation channel.

        A spectral calibration run produces data that can be conveniently be analyzed in the form of a matrix, $\mathbf{D}$, with R rows and C columns that contains the measured intensities in each

25   spectral channel/bin (the columns of the data matrix) as a function of time or frame/scan number (the rows of the data matrix). Each of the C columns represents an emission temporal profile for the corresponding spectral channel/bin. Each of the R rows represent the spectrum acquired during the corresponding data collection/acquisition period. The person of skill in art may devise numerous equivalent representations of the data obtained from a calibration run rather the

30   specific matrix described above, e.g. the components of the rows and columns may be transposed or the data may be manipulated without the use of a 2-D matrix. Each temporal profile contains

Peak Detection Transformation

Peak detection is performed on a total emission temporal profile. A preferred transformation to detect peaks is the slope of the total emission temporal profile, and is given as:

$$S_i = (I_{i+1} - I_i) + (I_{i+2} - I_{i-1}) \tag{1}$$

where S_i is the slope (as estimated by the detection transformation) at point I, and I_k is the intensity of the total emission temporal profile at point k. However, other peak detection transformations based on changes of intensity may also be used in the subject methods.

Statistical Distribution of Detection Transformation And Failure Analysis

The threshold parameter used in a peak detector may be an actual value for the slope. However, in a preferred embodiment of the invention the threshold is determined by the distribution of the peak detection transformation based on a probabilistic model An input variable is used to estimate the threshold. The detection transformations produce a parameter, for example S in Equation 1, that is used for peak detection. The performance of S in distinguishing baseline segments from peak segments in a temporal profile is highly influenced by the distribution of S when I is subjected to random variations only. The variance in S can be estimated by applying error propagation theory to Equation 1, and is given according to:

$$\sigma^2(S) = \Sigma \left\{ [\partial F(S)/ \partial I_k]^2 \, \sigma^2(I_k) \right\}$$

where F(S) is the detection transformation (Equation 1). For independent measurements, the above expression reduces to:

$$\sigma^2(S) = 4\sigma^2(I) \tag{2}$$

Thus, segments of a temporal profile that correspond to baselines with random variations are expected to produce amplified variations, according to Equation 2 , after the detection transformation.

The start of a peak is considered the first data point along the peak segment of the total emission profile that does not belong in the baseline population. The baseline segment's population produces a transformation distribution with a variance of $4\sigma^2(I)$ (Equation 2). The

-11-

Identification of the components of Multiple Color Calibration Standards

Calibration of fluorescent polynucleotide separation apparatus with various embodiments of the methods of the invention include the step of identification of the labeled polynucleotides of the multiple color calibration standards. The identification of the colored ladder fragments refers to the assignment of each labeled polynucleotide in a multiple color calibration standard to one of the peaks retained by the peak detector. Assignment can be accomplished by a variety of methods. Since the spectral calibration of fluorescent polynucleotide separation apparatus is accomplished under controlled conditions (known and prespecified materials and experimental parameters,) an efficient way to identify the labeled polynucleotides of the multiple color calibration standards is to take advantage of the controlled experimental conditions and the design of the colored ladder. For example, the multiple color spectral calibration standard design may be such that the fragment labeled with the dye DR110 in a multiple color calibration standard has the largest migration time. Under optimized and controlled experimental conditions, where the peak width and peak S/N ratio parameters allow multiple color calibration standard constituent polynucleotides to be detected and retained, the last peak would be the DR110-labeled fragment. A peak with such a high probability of being detected may serve as a reference peak to locate peaks corresponding to the other labeled polynucleotides of the multiple color calibration standard. Since the migration of a labeled DNA fragment is influenced primarily by the size of the DNA fragment, the labeling dye and the separation matrix, migration time offsets over a short migration interval are effective parameters to use in locating the peaks corresponding to the labeled polynucleotides of the multiple color calibration standards given the location of a reference peak such as the DR110-labeled peak.

If the mobilities of the labeled polynucleotides of the standard exhibit significant nonlinearities, and the migration of the colored ladder fragments is not easily (and reliably) predictable over a large range of migration times using offsets from one reference peak, the prediction range may be reduced by relying on offsets from neighboring peaks. For example, a polynucleotide labeled with DR110 may be used as a reference peak to locate the polynucleotide (in the same multiple color calibration standard mixture) labeled with DR6G. Subsequently, the polynucleotide labeled with DR6G (in the same standard) may serve as a reference peak to locate the polynucleotide labeled with DTAM. The polynucleotide labeled with DTAM (in the same standard) may then used to locate the polynucleotide labeled with DROX. Finally, the

-13-

information. Any spectrum acquired during any data collection/acquisition period can be estimated from the net analytical signals obtained in the spectral channels/bins. A spectrum is, thus, a background/baseline corrected row of **D**.

The dyes' reference spectra are, therefore, estimated from the corrected rows of **D** that correspond to data points along the peak segments of the total emission temporal profile. The peak maximum is the data point (row of **D**) recommended for estimating the dyes' reference spectra. Since the emission temporal profiles of the individual spectral channels/bins are not expected to be perfectly parallel, a row of **D** is corrected by estimating the net analytical signal in each spectral channel/bin using the peak detection information from the total emission temporal profile and appropriate search windows. Spectral calibration reference spectra are, also, normalized such that the maximum spectral intensity in each spectrum is set to equal 1. This is accomplished by dividing all corrected spectral intensities in each spectrum by the maximum corrected spectral intensity found in the spectrum.

Uncertainties in Dyes' Reference Spectra

The spectral intensity in a particular channel/bin of a normalized dye's reference spectrum can be expressed as:

$$R_i = I_i/I_m \tag{4}$$

where R_i is the normalized spectral intensity in the reference spectrum at the ith spectral channel/bin,

I_i is the net analytical signal in the ith spectral channel/bin, and

I_m is the highest net analytical signal in the spectrum.

The uncertainty in R_i is given according to:

$$\sigma^2(R_i)/R_i^2 = (\sigma^2/I_i^2)[1 + m^2] \tag{5}$$

where m is given as I_i/I_m, and

σ^2 the variance in the spectral intensities and is assumed to be equivalent in both spectral channels/bins.

The relative error in R_i may be expressed according to:

$$\sigma(R_i)/R_i = [1/SNR_i][1 + m^2]^{1/2} \tag{6}$$

-15-

programmable so as to facilitate modifications or the apparatus of the computer program may be in the form of "firmware" that is not readily subjected to modification.

Other embodiments of the invention include systems for calibrating a fluorescent polynucleotide separation apparatus. The calibration systems includes computer code that

5   receives a plurality of spectral and temporal data from a fluorescent polynucleotide separation apparatus. The system also comprises computer code that calculate a total emission temporal profile from the spectral and temporal data. The system may further comprise additional computer code for performing the subject methods of spectral calibration. Such additional code includes code for detecting peaks, and code for preparing spectral profile for each of the dyes

10   included in a calibration standard. As the computer code requires of the subject system requires a physical embodiment to function, the system also comprises a processor and computer readable medium (*e.g.* optical or magnetic storage medium) for storing the computer program code. The computer readable medium is functionally coupled to the processor.

Another aspect of the invention is methods and compositions for detecting the flow of

15   electrical current through a separation channel of a fluorescent polynucleotide separation apparatus. Such methods and compositions are particularly useful with fluorescent polynucleotide separation apparatus that employ multiple separation channels, *e.g.* a multi capillary or multiple microchannel system, because of interruptions in current flow in individual separation channels may be difficult to detect if a substantial percentage of the channels have

20   proper current flow. The subject electrical flow monitoring methods involve the use of fluorescent dyes that are spectrally distinct from fluorescently labeled polynucleotides of primary interest. These spectrally distinct fluorescent dyes are referred to herein as monitoring dyes. In a preferred embodiment of the invention, the monitoring dye is selected so as to produce significant emission when excited by the same excitation source or sources used to excite the

25   other fluorescent dyes in the composition of interest.

For example, a polynucleotide sequencing reaction product mixture (chain termination sequencing) may contain (1) four spectrally distinct fluorescent dyes, wherein each of the four dyes is correlated with a different polynucleotide base (*e.g.* fluorescently labeled dideoxy sequencing) and (2) a monitoring dye that is spectrally distinct from the four other dyes.

30   Movement of the monitoring dye in a separation channel can be used to confirm that current flow and therefore proper separation of the sequencing reaction products is occurring. Monitoring

be found in, among other places, Hermanson, <u>Bioconjugate Techniques</u>, Academic Press, San Diego, CA (1996). Unless indicated otherwise by context of usage, the term "monitoring dye" includes monitoring dye conjugates.

Embodiments of the invention include compositions comprising fluorescently labeled polynucleotides and one or more monitoring dyes, wherein the monitoring dyes are spectrally distinct from the other fluorescent dyes in the mixture. The monitoring dyes may be added to the composition either before, after, or during the formation of the fluorescently labeled polynucleotides for analysis. For example, a monitoring dye may be added to a polynucleotide sequencing reaction either before or after the reaction is terminated. In some embodiments of the invention, the subject compositions comprise multiple different monitoring dyes. In such embodiments, the monitoring dyes are preferably conjugates having different electrophoretic mobilities. In other embodiments of the subject compositions, a single signal fluorescent dye is present, but the dye molecules are conjugated to two or more different mobility modifier species so as to produce multiple opportunities to detect the monitoring dye during electrophoretic separation.

The invention also includes methods of detecting the flow of electrical current through a separation channel of a fluorescent polynucleotide separation apparatus by introducing a fluorescently labeled polynucleotide composition into a channel of a fluorescent polynucleotide separation apparatus. The fluorescently labeled polynucleotide composition comprises a polynucleotide labeled with a first fluorescent dye and a monitoring dye that is spectrally distinct from the first fluorescent dye. In most embodiments of the invention, the fluorescently labeled polynucleotide is a complex mixture of different length polynucleotides. Exemplary of such fluorescently labeled polynucleotide mixtures are the products of DNA sequencing reactions employing either fluorescently labeled primers or fluorescently labeled terminators, PCR amplification products formed by using fluorescently labeled primers, fluorescently labeled mini-sequencing reactions, products, fluorescently labeled oligonucleotide ligation reaction products, and the like. Such reactions produce genetic information that may be analyzed in the fluorescent polynucleotide separation apparatus. The monitoring dye is spectrally distinct from the fluorescent dyes used to label the polynucleotides that convey genetic information. For example, the invention includes a composition comprising a the complex mixture of different fluorescently labeled polynucleotides produced from four color chain termination sequencing and signal dye

-19-

CLAIMS

What is claimed is:

1. A method of calibrating a fluorescent polynucleotide separation apparatus, said method comprising the steps, introducing a fluorescent polynucleotide separation standard into said apparatus, wherein the standard comprises at least two polynucleotides of different length, each of the polynucleotides being labeled with a spectrally distinct fluorescent dye, separating the polynucleotides from each other, detecting the separated polynucleotides with a detector, wherein the detector collects spectral data from the separated polynucleotides over a plurality of spectral channels, collects and temporal data from the separated polynucleotides over a plurality of temporal points, and generating a total emission temporal profile from the spectral and temporal data.

2. The method according to **claim 1**, wherein the separation standard comprises four polynucleotide each of different length and labeled with a spectrally distinct dye.

3. The method of **claim 2**, wherein the length of the polynucleotides is selected so as minimize the spectral overlap of the fluorescent dye at each point of detection for the polynucleotides.

4. The method of **claim 1**, wherein the separation standard comprises five polynucleotide each of different length and labeled with a spectrally distinct dye.

5. The method according to **claim 1**, further comprising the step of detecting the peaks in the total emission temporal profile.

6. The method according to **claim 5**, further comprising the step of selecting a reference spectrum for each of the fluorescent dyes, wherein each reference spectrum substantially corresponds to peak of the emission temporal profile.

computer code that receives plurality of spectral and temporal data from a fluorescent polynucleotide separation apparatus, and computer code that calculates a total emission temporal profile from the spectral and temporal data.

5   14. A calibration standard for a fluorescent polynucleotide separation apparatus, standard comprising four polynucleotides of different length, each polynucleotide labeled with a different fluorescent dye having a distinctive spectral profile having a peak, wherein the length of each of the polynucleotides is such that the peak of the spectral profile of each dye does not significantly overlap between the separated fragment.

10

15. A kit for producing a calibration standard of **claim 14**, wherein the fluorescent labeled polynucleotides are stored in separate containers.

16. A method of detecting the flow of electrical current through a separation channel

15   of fluorescent polynucleotide separation apparatus, said method comprising the steps, introducing a fluorescently labeled polynucleotide composition to a channel of a fluorescent polynucleotide separation apparatus, said composition comprising a polynucleotide labeled with a first fluorescent dye, a monitoring dye that is spectrally distinct from the fluorescent dye, and detecting the monitoring dye.

20

17. The method according to **claim 16**, wherein the composition comprises a plurality of polynucleotides labeled with at least two spectrally distinct fluorescent dyes, wherein the monitoring dye is spectrally distinct from the fluorescent dyes.

25   18. The method of **claim 17**, wherein the polynucleotides labeled with at least two spectrally distinct fluorescent dyes is a polynucleotide sequencing reaction product mixture.

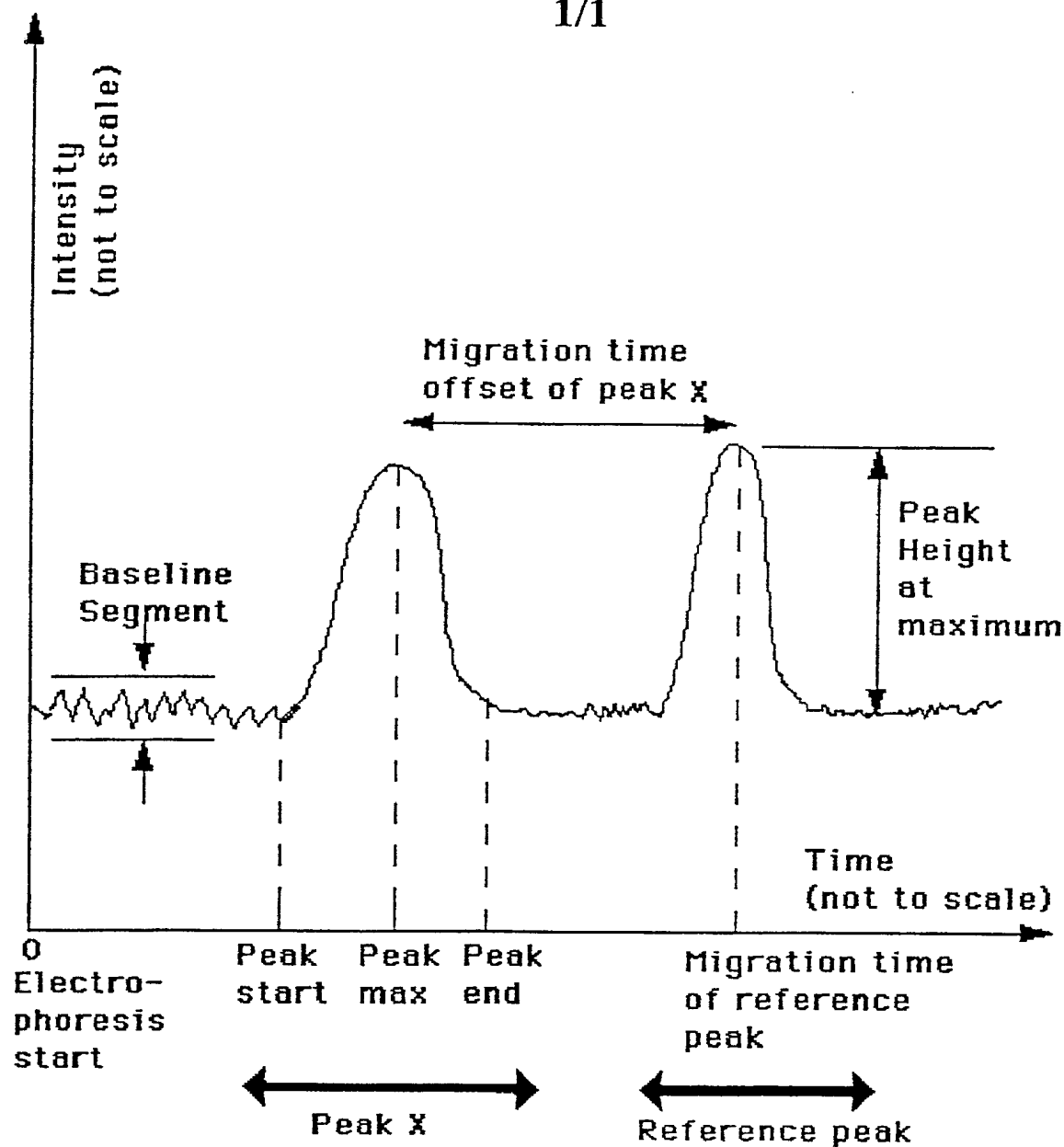19. The method of **claim 18**, wherein the monitoring dye is attached to a polynucleotide.

30

-23-

Figure 1

INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CONNELL C: "AUTOMATED DNA SEQUENCE ANALYSIS" BIOTECHNIQUES,US,NATICK, MA, vol. 5, no. 4, 1987, page 342-344,346-34 XP002043257 the whole document	1
A	WO 97 46963 A (PERKIN ELMER CORP) 11 December 1997 (1997-12-11) cited in the application the whole document	1